User Guide

XF Cell Mito Stress Test Kit

Reorder number 103015-100

For Research Use Only.

Use with XF^{e} and XF Extracellular Flux Analyzers.

Seahorse Bioscience

Product Description

The XF Cell Mito Stress Test is the standard assay for measuring mitochondrial function in cells. The XF Cell Mito Stress Test Kit is designed to evaluate mitochondrial function using the XF^{e} and XF Extracellular Flux Analyzers. The kit contains the reagents required to determine key parameters of mitochondrial function (*Refer the Definitions Box for more details).*

The ability to assess mitochondrial function has enabled researchers to advance their understanding of metabolism's key role in cellular physiology, disease pathology, and etiology. This assay provides insight into the cause of mitochondrial dysfunction and an in-depth understanding of metabolic pathways, signals, and phenotypes.

Introduction

The XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells (Figure 1).

The XF Cell Mito Stress Test uses modulators of respiration that specifically target components of the electron transport chain (ETC) in the mitochondria to reveal key parameters of metabolic function. The modulators (oligomycin, FCCP, and a mix of rotenone and antimycin A) are serially injected to measure ATP production, maximal respiration, and non-mitochondrial respiration, respectively. Proton leak and spare respiratory capacity are then calculated using these parameters and basal respiration.

As illustrated in Figure 2 and described in Table 1, each modulator targets a specific component of the ETC. Oligomycin inhibits ATP synthase (complex V) and the decrease in OCR following injection of oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. Carbonyl cyanide-4(trifluoromethoxy)phenylhydrazone (FCCP) is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the ETC is uninhibited and oxygen is maximally consumed by complex IV. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the cell's ability to respond to increased energy demand. The third injection is a mix of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of non-mitochondrial respiration driven by processes outside the mitochondria.

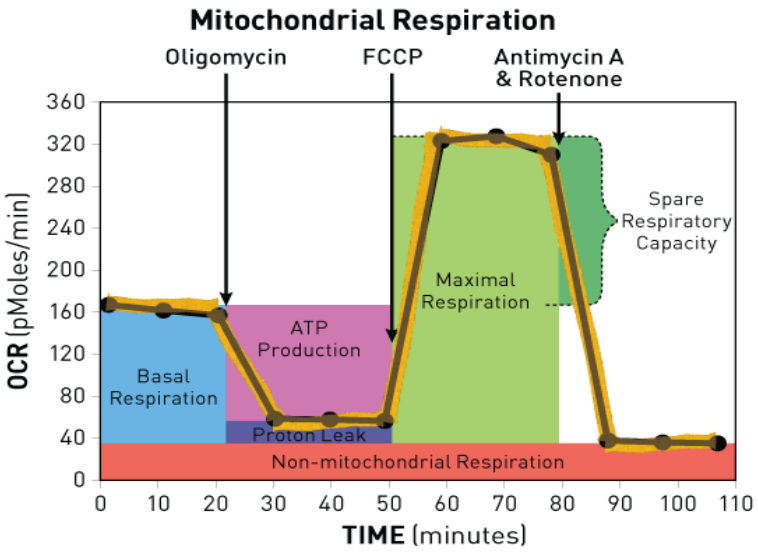


Figure 1: XF Cell Mito Stress Test profile of the key parameters of mitochondrial respiration. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration.

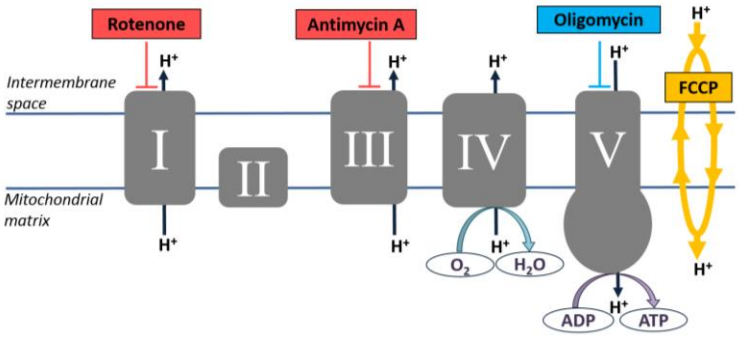


Figure 2: XF Cell Mito Stress Test Modulators of the ETC. This diagram illustrates the complexes of the ETC and the target of action of all of the compounds in the XF Cell Mito Stress Test Kit. Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, and rotenone and antimycin A inhibit complexes I and III, respectively.

Table 1: **XF Cell Mito Stress Test Reagents** (in order of injection)

Compound(s)	ETC Target	Effect on OCR
Oligomycin	ATP Synthase Complex V	Decrease
FCCP	Inner Mitochondrial Membrane	Increase
Rotenone and Antimycin A	Complex I and III (respectively)	Decrease

Kit Contents

The XF Cell Mito Stress Test Kit includes 6 foil pouches that each contain reagents sufficient for a complete XF Cell Mito Stress Test in either the 96 or 24 well XF Cell Culture Microplate. Every pouch includes one tube of each of the following compounds: oligomycin, FCCP and a mix of rotenone/antimycin A as indicated below:

Compound	Cap Color	Quantity per tube (nmol)
Oligomycin	Blue	63
FCCP	Yellow	72
Rotenone + antimycin A	Red	27 (of both)

Kit Storage

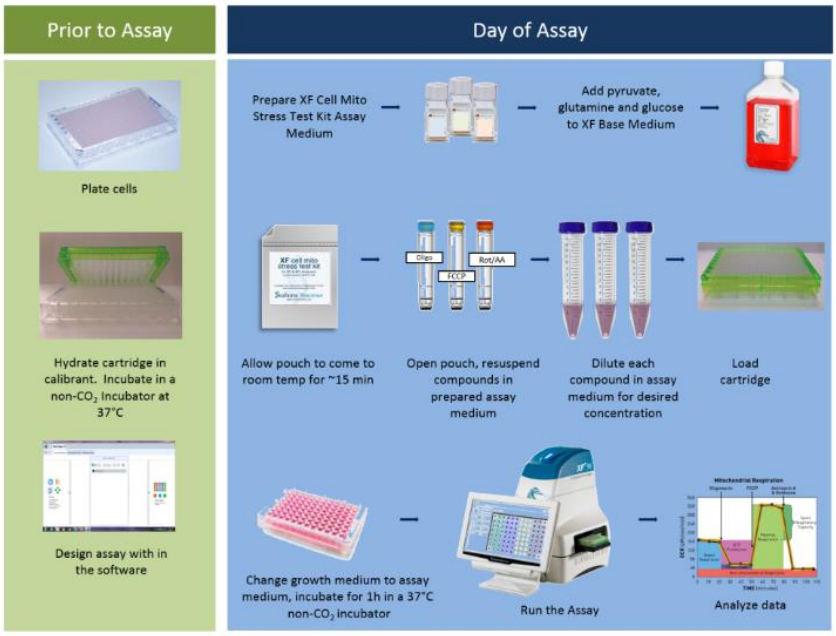
Product ships at ambient temperature.
Product should be stored at -20°C upon arrival.

Additional Required Items

XF^e/XF96 or24 Analyzer	Seahorse Bioscience	
XF Base Medium	Seahorse Bioscience	102353-100
100 mM Pyruvate	Sigma	S8636 or equivalent
200 mM Glutamine	Sigma	G8540 or equivalent
2.5 M Glucose	Sigma	G8769 or equivalent

Narrow p1000 pipette tips are recommended for reconstituting compounds within the tubes provided (e.g. Fisherbrand™ SureOne™ Micropoint Pipet Tips, catalog #: 02-707-402).

Figure 3: XF Cell Mito Stress Test Assay Workflow


Prior to Assay
Day of Assay
Plate cells
Hydrate cartridge in calibrant. Incubate in a non-CO2 incubator at 37°C
Design assay with in the software
Prepare XF Cell Mito Stress Test Kit Assay Medium
Add pyruvate, glutamine and glucose to XF Base Medium
Allow pouch to come to room temp for ~15 min
Open pouch, resuspend compounds in prepared assay medium
Dilute each compound in assay medium for desired concentration
Load cartridge
Change growth medium to assay medium, incubate for 1h in a 37°C non-CO2 incubator
Run the Assay
Analyze data

GLOSSARY

Basal Respiration
Oxygen consumption used to meet cellular ATP demand and resulting from mitochondrial proton leak. Shows energetic demand of the cell under baseline conditions.

ATP Production
The decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin represents the portion of basal respiration that was being used to drive ATP production. Shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell.

H+ (Proton) Leak
Remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production.

Maximal Respiration
The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve.

Spare Respiratory Capacity
This measurement indicates the capability of the cell to respond to an energetic demand as well as how closely the cell is to respiring to its theoretical maximum. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility.

Non-mitochondrial Respiration
Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.

Workflow

Day Prior to Assay

1. Turn on the XF^{e}/XF Analyzer and warm up overnight (minimum 5 hours).
2. Plate cells at a previously determined optimized density in the XF Cell Culture Microplate using the appropriate cell culture growth medium. Refer to Basic Procedure "Seeding Cells in XF Cell Culture Microplates" available on the Seahorse Bioscience website for more information. www.seahorsebio.com/resources/documentation/basicprocedures
3. Hydrate a sensor cartridge in XF Calibrant at 37°C in a non-CO_2 incubator overnight. Refer to Basic Procedure "Hydrating the Sensor Cartridge" on the Seahorse website for more details. www.seahorsebio.com/resources/documentation/basicprocedures
4. Design experiment in Wave or XF software. Use the default software settings. See the instrument user manual for additional details.

Day of Assay

Prepare Assay Medium

1. Prepare assay medium by supplementing XF Base Medium. Seahorse recommends 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose as a starting point; however, desired medium composition can be varied depending on cell type or *in vitro* culture conditions. Refer to Basic Procedure "Preparing Assay Media for Use XF Assays" on the Seahorse Bioscience website for more information. www.seahorsebio.com/resources/documentation/basicprocedures
2. Warm assay medium to 37°C.
3. Adjust pH to 7.4 with NaOH (*Note: Seahorse recommends sterile filtration following pH adjustment).*
4. Keep at 37°C until ready to use.

Prepare Stock Compounds

Important: *Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.*

1. Remove foil pouch from XF Cell Mito Stress Test Kit box. Each pouch contains reagents sufficient for a complete XF Cell Mito Stress Test in a 96 or 24 well XF Cell Culture Microplate.
2. Allow compounds to warm to room temp in the sealed pouch for approximately 15 minutes.
3. Open pouch and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap). Place tubes in a small tube rack.
4. Resuspend contents of each tube with prepared assay medium in volumes described in Table 2 with a p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds.

A NOTE ON OPTIMIZATION

Optimal concentrations of FCCP and cell seeding density should be empirically determined for your cells prior to the assay. Refer to **FCCP Optimization with the XF Cell Mito Stress Test**, and **Seeding Cells in XF Cell Culture Microplates** Basic Procedures available on the Seahorse website for more information on optimization.

The Cell Line Reference Database (www.seahorsebio.com/learning/cell-line.php) is a good resource for finding reference publications for your cell type of interest.

If you are optimizing concentrations for your assay, start with the highest concentrations given in Table 3 and make serial dilutions to cover the desired concentration range.

Table 2: Stock Solutions

	Volume of Assay Medium	Final Concentration
Oligomycin	630 µL	100 µM
FCCP	720 µL	100 µM
Rotenone / Antimycin A	540 µL	50 µM

Prepare Compounds for Loading in Sensor Cartridge

There are two approaches to loading the injection ports of the sensor cartridge:

Constant loading volume/ variable compound concentration
This approach entails loading a constant volume of compound in each injection port and requires that each compound be prepared at a different concentration.

OR

Constant compound concentration/ variable loading volume
This approach entails preparing the compounds at a constant concentration and requires that a different volume of each compound be loaded in the injection port.

Table 3 describes how to prepare to load the cartridges using both options.

Prepare 3 mL of each compound in assay medium according to the approach you have chosen. ***Seahorse recommends using 1 µM of oligomycin for most cells. Please contact Technical Support with any questions.***

Table 3: Compound Preparation for Loading Sensor Cartridge Ports

XF^e/XF 96		Constant Volume Starting well volume: 175 µL assay medium				Constant Concentration Starting well volume: 180 µL assay medium			
Port A Oligomycin	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**8X [Port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [Port] (µM)**	**Add to port (µL)**
	0.5	120	2,880	4	25	150	2,850	5	20
	1.0	240	2,760	8	25	300	2,700	10	20
	2.0	480	2,520	16	25	600	2,400	20	20
Port B FCCP	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**9X [Port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [Port] (µM)**	**Add to port (µL)**
	0.125	33.8	2,966.2	1.125	25	37.5	2,962.5	1.25	22
	0.25	67.5	2,932.5	2.25	25	75	2,925	2.5	22
	0.5	135	2,865	4.5	25	150	2,850	5	22
	1.0	270	2,730	9	25	300	2,700	10	22
	2.0	540	2,460	18	25	600	2,400	20	22
Port C Rotenone/ Antimycin A	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [Port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [Port] (µM)**	**Add to port (µL)**
	0.5	300	2,700	5	25	300	2,700	5	25

XF^e/XF 24		Constant Volume Starting well volume: 525 µL assay medium				Constant Concentration Starting well volume: 500 µL assay medium			
Port A Oligomycin	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**8X [port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [port] (µM)**	**Add to port (µL)**
	0.5	120	2,880	4	75	150	2,850	5	56
	1.0	240	2,760	8	75	300	2,700	10	56
	2.0	480	2,520	16	75	600	2,400	20	56
Port B FCCP	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**9X [port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [port] (µM)**	**Add to port (µL)**
	0.125	33.8	2,966.2	1.125	75	37.5	2,962.5	1.25	62
	0.25	67.5	2,932.5	2.25	75	75	2,925	2.5	62
	0.5	135	2,865	4.5	75	150	2,850	5	62
	1.0	270	2,730	9	75	300	2,700	10	62
	2.0	540	2,460	18	75	600	2,400	20	62
Port C Rotenone/ Antimycin A	**[Final well] (µM)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [port] (µM)**	**Add to port (µL)**	**Stock volume (µL)**	**Media volume (µL)**	**10X [port] (µM)**	**Add to port (µL)**
	0.5	300	2,700	5	75	300	2,700	5	69

Load Sensor Cartridge

*Refer to the Basic Procedure, "**Loading the Sensor Cartridge with Compounds**" on the Seahorse Bioscience website for proper port-loading technique.*
www.seahorsebio.com/resources/documentation/basicprocedures/index.php

1. Standard Assay - no additional injection
   Load compounds into the appropriate ports of a hydrated sensor cartridge:

   Port A: Oligomycin
   Port B: FCCP
   Port C: Rotenone/antimycin A

2. Modified Assay – additional injection included
   To inject an additional compound prior to oligomycin, use port A for the desired compound and then load:

   Port B: Oligomycin
   Port C: FCCP
   Port D: Rotenone/antimycin A

Table 4 lists the appropriate volumes and concentrations for this injection scheme.

Table 4: Compound Injection Volumes Involving an Acute Injection.

	XF^e/XF 96				XF^e/XF 24			
	Constant Volume Starting well volume: 175 µL assay medium		**Constant Concentration Starting well volume: 180 µL assay medium**		**Constant Volume Starting well volume: 525 µL assay medium**		**Constant Concentration Starting well volume: 500 µL assay medium**	
Port	Vol.	Conc.	Vol.	Conc.	Vol.	Conc. Vol.	Vol.	Conc.
A	25 µL	8X	20 µL	10X	75 µL	8X	56 µL	10X
B	25 µL	9X	22 µL	10X	75 µL	9X	62 µL	10X
C	25 µL	10X	25 µL	10X	75 µL	10X	69 µL	10X
D	25 µL	11X	27 µL	10X	75 µL	11X	75 µL	10X

Prepare XF Cell Culture Microplate for Assay

1. Remove cell culture microplates from 37°C CO_2 incubator and examine cells under microscope to confirm confluence.
2. Remove assay medium from water bath.
3. Change the cell culture growth medium in the cell culture microplate to warmed assay medium using a multichannel pipette and place the cell culture microplate into a 37°C non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.

Run the XF Cell Mito Stress Test

Open software and retrieve saved assay template file. Follow instructions below for your specific software:

If you are using XF Software:

1. Browse for and open the saved design file then click “Run”.
2. Place the calibration plate with the loaded sensor cartridge on the instrument tray. Calibration will take approximately 15-30 minutes.
   Note: Remove cartridge lid and verify correct plate orientation.
3. When prompted, replace the calibration plate with the cell culture microplate then click “Start”.

If you are using Wave:

1. Browse and open the saved design file, select the “Review and Run” tab, and then click “Start Run”.
2. When prompted, place the loaded sensor cartridge with the calibrant plate into the instrument, then click “I’m ready”. Calibration will take approximately 15-30 minutes.
   Note: Remove cartridge lid and verify correct plate orientation.
3. Following calibration and equilibration of the cell culture microplate, when prompted click “I’m ready”. Load the cell culture microplate and click “I’m ready” to run the assay.

Data Analysis

The XF Mito Stress Test Report Generator automatically calculates the XF Cell Mito Stress Test parameters from Wave data that has been exported to Excel. It can be used with either a standard or modified stress test protocol and provides a convenient, customizable, one-page assay summary. The Report Generator can be installed either alongside Wave or directly from the Seahorse Bioscience website. Visit www.seahorsebio.com/support/software/stress-test-generator.php to learn more about the XF Stress Test Report Generators and download the User Guide.